Archives of Biochemistry and Biophysics 637 (2018) 64–72

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Atorvastatin affects negatively respiratory function of isolated endothelial mitochondria

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ARTICLE INFO

Keywords:
Atorvastatin
Pravastatin
Endothelial mitochondria
Respiration
Membrane potential
Reactive oxygen species

ABSTRACT

The purpose of this research was to elucidate the direct effects of two popular blood cholesterol-lowering drugs used to treat cardiovascular diseases, atorvastatin and pravastatin, on respiratory function, membrane potential, and reactive oxygen species formation in mitochondria isolated from human umbilical vein endothelial cells (EA.hy926 cell line). Hydrophilic pravastatin did not significantly affect endothelial mitochondria function. In contrast, hydrophobic calcium-containing atorvastatin induced a loss of outer mitochondrial membrane integrity, an increase in hydrogen peroxide formation, and reductions in maximal (phosphorylating or uncoupled) respiratory rate, membrane potential and oxidative phosphorylation efficiency. The atorvastatin-induced changes indicate an impairment of mitochondrial function at the level of ATP synthesis and at the level of the respiratory chain, likely at complex I and complex III. The atorvastatin action on endothelial mitochondria was highly dependent on calcium ions and led to a disturbance in mitochondrial calcium homeostasis. Uptake of calcium ions included in atorvastatin molecule induced mitochondrial uncoupling that enhanced the inhibition of the mitochondrial respiratory chain by atorvastatin. Our results indicate that hydrophobic calcium-containing atorvastatin, widely used as anti-atherosclerotic agent, has a direct negative action on isolated endothelial mitochondria.

1. Introduction

Statins, commonly used cholesterol-lowering drugs, are perceived to have a positive tolerability profile and have a well-documented benign influence on the cardiovascular system [1–3]. Statins act by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.88), an enzyme that catalyzes the first committed step of the mevalonate pathway, and consequently, statins inhibit endogenous cholesterol synthesis. On the other hand, statins also inhibit the synthesis of mevalonate, a precursor to heme *a* (a structural part of cytochrome *c* oxidase) and coenzyme Q10 (CoQ10, or ubiquinone), which are obligatory components of the mitochondrial electron transport chain [1]. CoQ10 is both an essential electron carrier and an important antioxidant in the mitochondrial inner membrane [4]. It is well documented that a decreased level of CoQ10 may compromise mitochondrial function by impairing ATP production and augmenting oxidative stress [5–10]. Deficiency of CoQ10 has been proposed as a main reason for muscle mitochondrial dysfunction in patients treated with statins [11,12]. Besides CoQ10 deficiency, statins are proposed to damage mitochondria via inhibition of mitochondrial respiratory chain complexes, diminution of ATP synthesis, elevation of reactive oxygen species (ROS) formation, and induction of the mitochondrial apoptosis pathway [6,13]. Most of these disturbances have been observed in studies related to statin-associated myopathies. Thus, statin-induced mitochondrial dysfunction is a complex mechanism and it is not fully understood, especially in statin-induced endothelial dysfunction.

Mitochondria regulate various cellular processes. The role of these organelles goes beyond their capacity to create molecular fuel (i.e., ATP) and generate reactive oxygen species (ROS). Endothelial cells lining the lumen of blood vessels have permanent contact with blood-transported compounds, including statins. Therefore, endothelial mitochondria are biosensors for drug-induced toxicity. In comparison with other mammalian cell types with higher energy requirements, endothelial cells have a low mitochondrial content and obtain most of their energy from anaerobic glycolysis [14–17]. However, endothelial mitochondria are crucial for maintaining a fine regulatory balance between mitochondrial Ca^{2+} uptake and the generation of NO and ROS, which, besides contributing to oxidative stress and cell death, participate in cell signaling [16,18–20]. Endothelial mitochondria dysfunction contributes to the development of nearly all vascular diseases.

Abbreviations: CoQ10, coenzyme Q10; mΔΨ, mitochondrial membrane potential; OXPHOS, oxidative phosphorylation; RCR, respiratory control ratio; ROS, reactive oxygen species

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https://doi.org/10.1016/j.abb.2017.12.003
Received 18 August 2017; Received in revised form 27 November 2017; Accepted 2 December 2017
Available online 05 December 2017
0003-9861/

Due to the well-documented positive effects on the cardiovascular system, statins are commonly applied drugs to reduce the development of cardiovascular diseases. Statins stabilize atherosclerotic plaques, reduce the inflammatory response, and improve the functioning of vascular endothelium [21–23]. Although the anti-atherosclerotic effect of statins is well documented, in some cases, the usage of statins may lead to endothelial dysfunction [24–28]. It has been hypothesized that statin-induced mitochondrial dysfunction may be associated with endothelial dysfunction [29,30]. It has been reported that mitochondrial dysfunction induced by statin contributes to endothelial dysfunction in a significant portion of patients with coronary artery disease [29]. Such data suggest the existence of statin-induced endothelial mitochondria-related side effects, like in the case of statin-associated muscle mitochondria-related adverse effects in myopathies.

Studies on the influence of statins on endothelial dysfunction have mainly utilized biological material derived from patients treated with statins or from animals. To understand the direct effects of statins on endothelial mitochondria, in this study, we used mitochondria isolated from endothelial cells. Depending of the hydrophobicity/hydrophilicity, statin molecules can affect cells and mitochondria in different ways [8]; thus, we have chosen a hydrophobic statin, atorvastatin, and a hydrophilic statin, pravastatin. Both statins are suitable for in vitro investigations because they are not inactive prodrugs requiring activation. The aim of this work was to elucidate the direct effects of atorvastatin and pravastatin on respiratory function, membrane potential (mΔΨ), and ROS formation of endothelial mitochondria isolated from EA.hy926 cells.

2. Material and methods

2.1. Chemicals

All chemicals used in the experiments, including pravastatin (P4498) and atorvastatin (1044516), were purchased from Sigma-Aldrich. Pravastatin was dissolved in water, and atorvastatin was dissolved in methanol. Control measurements (without statins) with a given amount of appropriate solvent were performed and taken into account for calculations.

2.2. Cell culture and mitochondria preparation

We used the stable human endothelial cell line EA.hy926 (ATCC® CRL-2922™), which was originally derived from a human umbilical vein [31]. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 2% hypoxanthine-aminopterin-thymidine (HAT), and 1% penicillin/streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. The EA.hy926 cells were cultured in 140-mm dishes until they reached approximately 95–100% confluence. Cells that were used in this study were between passages 4 and 12.

After 6 days of cultivation, the cells were harvested with trypsin/EDTA, rinsed twice with phosphate-buffered saline (PBS), containing 10% and 5% FBS, respectively, and centrifuged at 1200 x *g* for 10 min at 4 °C. Subsequently, the cells were washed in cold PBS medium and then centrifuged again. The final cell pellet was resuspended in PBS (1 g of cells per 3 ml) and kept on ice. Cells (3.75 ± 0.49 g, mean ± SD, $n = 8$) were harvested from 80 dishes of culture for each experiment.

Mitochondria were isolated from EA.hy926 cells using a very efficient isolation procedure that produces highly active and well-coupled mitochondria [32]. As modification, incubation media contained 5 mM Tris/HCl and 10 mM HEPES (pH 7.2) instead of 15 mM Tris/HCl (pH 7.2). The final mitochondrial pellet was resuspended in medium containing 0.25 M sucrose, 5 mM Tris/HCl, and 10 mM HEPES (pH 7.2). The yield of the isolated mitochondria was equal to 3.2 ± 0.7 mg of mitochondrial protein per g of cells (mean ± SD, $n = 10$).

2.3. Measurements of mitochondrial respiration and membrane potential

Mitochondrial respiration and mΔΨ were measured in isolated endothelial mitochondria as previously described [32]. Oxygen uptake was determined polarographically using a Rank Bros. (Cambridge UK) oxygen electrode or a Hansatech oxygen electrode with either 2 mg or 0.5 mg of mitochondrial protein in either 2.8 ml or 0.7 ml of standard incubation medium (at 37 °C), respectively, which consisted of 150 mM sucrose, 2.5 mM KH_2PO_4, 2 mM $MgCl_2$, 20 mM Tris/HCl (pH 7.2), and 0.1% BSA. O_2 uptake values are presented in nmol $O_2 \times min^{-1} \times mg^{-1}$ protein. Membrane potential was measured simultaneously with oxygen uptake using a tetraphenylphosphonium (TPP^+)-specific electrode. The values for mΔΨ are given in mV.

Succinate (7 mM, plus 2 μM rotenone) or malate (7 mM) were used as respiratory substrates,. All measurements were performed in the presence of 0.05 mM ATP to activate the substrate dehydrogenases. Phosphorylating respiration (state 4) was measured using 150 μM (pulse) or 1.2 mM (saturating) ADP, and uncoupled respiration (state U) was measured using up to 0.8 μM FCCP. Oxidative phosphorylation (OXPHOS) efficiency was assessed by calculation of coupling parameters, i.e., ADP/O ratio and respiratory control ratio (RCR). Non-phosphorylating (resting state, state 4) respiration measurements were performed in the absence of exogenous ADP and in the presence of 1.8 μM carboxyatractyloside and 0.5 μg/ml oligomycin, which inhibit the activities of the ATP/ADP antiporter and ATP synthase, respectively. Only high-quality mitochondria preparations, i.e., with ADP/O values of approximately 2.3 and 1.3 and RCR of approximately 4.0–4.3 and 2.6–3.0 with malate and succinate as the respiratory substrates, respectively, were used in the experiments.

2.4. Measurement of mitochondrial enzyme activities

The activity of citrate synthase was determined by spectrophotometrically tracking the formation of DTNB-CoA at 412 nm using a UV 1620 Shimadzu spectrophotometer as described previously [32]. The activity of citrate synthase was measured in 0.7 ml of reaction medium containing 100 mM Tris/HCl (pH 8.0) 100 μM acetyl CoA, 100 μM 5,5′-di-thiobis-(2-nitrobenzoic acid) (TNB), 0.1% Triton X-100, and 100 μM oxaloacetate, with 50 μg of mitochondrial protein.

The maximal activity of cytochrome *c* oxidase and the integrity of the outer mitochondrial membrane were assessed polarographically as described previously [32]. The activity was assessed in 0.7 ml of the standard incubation medium (*point 2.3*) with 0.5 mg of mitochondrial protein without an exogenous respiratory substrate and in the presence of sequentially added antimycin A (10 μM), 8 mM ascorbate, 0.06% cytochrome *c*, and up to 2 mM N,N,N'N'-tetramethyl-*p*-phenylenediamine (TMPD).

All enzymatic assays were performed at 37 °C with continuous stirring.

2.5. Measurement of H_2O_2 production in isolated mitochondria

Mitochondrial H_2O_2 production was measured by the amplex red-horseradish peroxidase method (Invitrogen). Fluorescence was kinetically monitored for 10 min at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using an Infinite M200 PRO Tecan multimode reader. Mitochondria (0.1 mg mitochondrial protein) were incubated in 0.5 ml of the standard incubation medium (*point 2.3*) with 0.1 U/ml horseradish peroxidase, 5 μM amplex red reagent and 7 mM succinate (plus 2 μM rotenone) or 7 mM malate as oxidizable substrates. H_2O_2 production in the uncoupled state was measured using 0.8 μM FCCP. For measurements in the phosphorylating state we used 1.2 mM ADP. H_2O_2 production rates were determined from slopes calculated from readings obtained in repeated measurements.